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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The goal of this exploratory study is to determine whether bio-available iron (BAI) contributes, synergistically with estrogens, to breast cancer (BC), particularly in older women. We hypothesize that an accumulation of a high BAI level is one of the pre-neoplastic changes in post-menopausal women, and in the presence of estrogens, it is BAI that increases risk of BC. We will test our hypothesis using blood samples collected by the New York University Women's Health Study. BAI in sera of 30 subjects who were apparently healthy at the time of blood donation but diagnosed with BC ½-6 years later and matched controls (2 controls per case) will be examined. BAI will be determined using fluorescent calcein as an iron chemosensor. We will use the same subjects as in the previous study, which has shown a positive association between post-menopausal serum levels of estradiol and breast cancer. Unconditional logistic regression models will be used to determine whether iron and estradiol act in synergy. If our hypothesis is confirmed, then an early detection and perhaps prevention of BC will be possible, as well as mechanisms of BC can be proposed.

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Introduction:

Of the 175,000 new BC cases estimated for 1999, over 82,000 were women ≥ 65 years old. BC incidence rate in women ≥ 65 was 441 per 100,000 compared to 75 per 100,000 for women under 65 years of age. Yet, there is insufficient information on biological mechanisms affecting the onset and progression of BC in older women. Now, there is an even greater need to address BC etiology in older women, because of the baby boomer generation entering this age range. One of the physiological differences between young and old women is that, in young women, iron stores remain low during child-bearing years due to loss of menstrual blood. Ferritin, an iron storage protein, remains low until menopause, and then plateaus in the 80 μg/l range (1). Serum iron, which is the iron in transferrin, total iron-binding capacity of transferrin, percentage saturation of transferrin, as well as ferritin have been used as biological indices of body iron storages in epidemiological studies. However, iron ions trapped in ferritin and transferrin are not readily bioavailable for adverse health effects. *Therefore, measuring ferritin and transferrin may not be a direct measurement of the toxic form of iron, which is the bioavailable iron (BAI).* Perhaps for these reasons, the role of iron in human cancer is inconclusive by epidemiological studies (2-8).

Development of a sensitive fluorescent method for the detection of BAI in serum:

The labile iron pool of cells constitutes a cytosolic fraction of low molecular weight (LMW) chelators bound iron or BAI, which can be characterized as iron (a) exchangeable and chelatable; (b) easily bioavailable for uptake by ferritin, heme, transferrin, or chelators; (c) metabolically and catalytically reactive for oxidant formation and likely responsible for iron toxicity and carcinogenicity; (d) possibly having regulatory properties which may affect iron responsive element-binding protein activity (9, 10). *Levels of BAI in serum have never been reported or estimated.* Although several methods have been introduced for the detection of non-transferrin bound iron (NTBI) (11, 12), this fraction of iron includes not only LMW iron, but also iron bound to other proteins such as albumins, which is neither redox active nor bioavailable.

Calcein (CA) as a fluorescence probe was used for measuring LMW iron in cells (13-17). The principle of the test is based on the binding of CA to LMW iron, in a stoichiometric manner, which results in a fluorescence quenching. However, we found that this fluorescent CA assay has some technical disadvantages when applied to serum, *i.e.* the CA fluorescence can be quenched by compounds in the serum, which are not iron-related. Such interference can greatly affect the specificity of the assay and its determination of iron levels. We have improved the specificity and the sensitivity of the assay by adding an iron chelator deferoxamine (DFO) into the samples. By filtering the sample through an Ultra-free membrane with a nominal molecular weight limit (NMWL) of 10 kDa, the iron present in the filtrates is LMW-bound. After optimizing the experimental conditions, we have shown that CA can detect LMW iron at a detection limit of 0.02 μM, at least 50 times more sensitive than any colorimetric method. We have further shown that levels of LMW iron ranged from 0.08 μM to 0.19 μM in the sera of 6 normal, healthy male subjects. Freeze-thawing up to 5 times did not affect the levels of BAI in the sera. This has been resulted in a publication (Appendix A) (18).

Changes in study design:

As originally proposed in this award to study 30 breast cancer cases and 60 matched controls, we would have two shortcomings: 1) without testing the reliability of the methods for the measurements of

the markers; 2) a very limited statistical power to detect significance, if any. These two shortcomings could result in a misuse of valuable breast cancer case serum samples. Instead, we have chosen 40 cancer-free subjects with 120 serum samples over a 2 year-interval (3 samples per subject) for reliability studies of transferrin and ferritin. Since one of the organ targets by iron metabolism is the kidney, we have used renal cell carcinoma (RCC) rather than breast cancer as our study model.

Reliability studies of serum iron, ferritin, and nitrite:

Reliability coefficient is the proportion of the variance due to between-subject variability and indicates how well a single measurement represents the long-term average level within an individual. New York University (NYU) Women's Health Study participants who had given blood on three or more occasions with a yield of 11 or more aliquots per visit, who had not been diagnosed with cancer or cardiovascular disease, and who had not been selected as a control in any case-control study nested within the cohort were eligible for the reliability study. One hundred-twenty serum samples collected at yearly intervals from 40 healthy women (20 pre-menopausal and 20 post-menopausal) were used for the reliability analysis. Levels of serum iron, ferritin, calcium (Ca), nitrite (NO_2^-), and total nitrite ($NO_2^- + NO_3^-$) were initially measured in 45 serum samples from 15 subjects (8 pre-menopausal women and 7 post-menopausal). The reliability coefficients (RCs) for serum iron, ferritin, Ca, nitrite, and total nitrite were 0.47, 0.77, 0.33, 0.51, and 0.06, respectively. For parameters with RCs>0.40, the study was extended to a total of 40 subjects in order to increase the precision of the reliability estimates. Ca and total nitrite were not studied any further.

The reliability was estimated by the intraclass correlation coefficient. Variance components were obtained in an ANOVA analysis assuming a one-way random effects model. Reliability coefficients (RCs) were computed using the log-transformed data. Exact 95% confidence intervals (CI) were calculated.

Data on this study were submitted for publication (Appendix B) (19). Average levels at first visit of serum iron, ferritin, and nitrite were higher in post-menopausal women than in pre-menopausal women, and the difference was statistically significant for serum iron ($p<0.05$) and approaching significance for ferritin ($p<0.08$). At visits two and three, the same trends were observed (data not shown). The RCs for the three parameters measured in the sera of all 40 subjects, confirming relatively high RCs for ferritin and nitrite as previously obtained using sera from 15 of the 40 subjects. Ferritin has the highest RCs, followed by nitrite, with higher RCs in pre-menopausal than in post-menopausal women. In contrast, serum iron has a low RC, particularly in pre-menopausal women. This may be due to the variation in blood loss by menstrual cycling in pre-menopausal women.

Nested case-control study:

Cases of RCC were identified through the active follow-up of the cohort by questionnaires mailed approximately every 2 years and telephone interviews for non-respondents, as well as by record linkages with the state-wide cancer registries in New York, New Jersey, Connecticut, and Florida and with the U.S. National Death Index. Medical and pathology reports were requested to confirm the diagnosis. For each case subject, 2 controls were selected at random from the risk set of women who were alive and free of disease at the time of diagnosis of the case (index date), and who matched the case on menopausal status at the enrollment, date of birth (± 6 months), and number (1, > 1) of blood donations.

Data on this study were submitted for publication (Appendix B) (19). Serum levels of iron, ferritin, and nitrite were then measured in 24 women who developed renal cell carcinoma (RCC) after enrollment in the cohort and 48 controls matching the cases on the age, menopausal status, and time of blood donation. The mean age (±SD) of the study population at blood donation was 56.9 years (±6.3 years). The ethnic distribution was as follows: 67% Caucasians, 8% African-Americans, and 25% others. The mean body mass index was 25.4 kg/m^2 (±3.6 kg/m^2) for case participants and 25.2 kg/m^2 (±4.0 kg/m^2) for control participants. Among the cases, the median lag time between blood donation and diagnosis of RCC was 6.6 years (range, 1.8-12.2 years). There was a higher proportion of ever smokers in cases (53%) than in controls (37%) with smoking status missing for 5 cases and 7 controls. We have found that levels of serum iron, ferritin, and nitrite were slightly lower in the case than in the control subjects, but the differences were not statistically significant. In conditional logistic regression analyses, higher levels of ferritin appeared to be associated with a decrease in risk [adjusted odds ratio (OR) = 0.55 in the above median *vs* the below median] and nitrite (OR = 0.52 in the above median *vs* the below median) after adjustment for smoking and body mass index (Table 4). A slight increase in risk appeared for serum iron (OR = 1.12 in the above median *vs* the below median).

Key research accomplishments:

1) Developed a sensitive fluorescent method for the detection of BAI, which is the most likely active form of iron in iron-induced toxicity and carcinogenicity;

2) Validated three potentially useful markers of iron indices, such as serum iron and ferritin;

3) Tested an association of serum iron, ferritin, and nitrite with the risk of RCC in women.

Reportable outcomes:

Publication list:

Ali, A. M., Zhang, Q., Dai, J. S., and Huang, X. "Calcein as a fluorescent iron chemosensor for the determination of low molecular weight iron in biological fluids." *Biometals*, in press, 2002.

Ali, M. A., Akhmedkhanov, A., Jaquotte-Zeleniuch, A., Toniolo, P., Frenkel, K., and Huang, X. "Reliability of serum iron, ferritin, and nitrite, and association with risk of renal cancer in women." Submitted to *Cancer Epidemiol. Biomark. Prev.*

Abstracts:

Ali, M. A., Akhmedkhanov, A., Zeleniuch-Jaquotte, A., Toniolo, P., and **Huang, X.** 93rd Annual Meeting of American Association for Cancer Research, April 2002, San Francisco, CA. "Serum ferritin, iron, nitrite and risk of renal cancer in women." *Proc. Am. Assoc. Cancer Res.*, **43**, p1040, 2002.

Personnel:

Xi Huang, Ph.D. Principal Investigator, 10% effort;
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Conclusions:

In summary, calcein is a sensitive fluorescent iron chemosensor, which can be applied for the measurements of LMW iron. The assay is simple and can be iron specific. Ferric citrate is probably the most appropriate iron standard for CA fluorescence quenching. The differences in fluorescence reading between the same sample ±DFO are recommended for iron quantification. After optimizing the experimental conditions, the sensitivity of the calcein assay is 0.02 μM of iron.

Our studies indicate that serum iron has a low reliability coefficient, particularly in pre-menopausal women. With greater variability of serum iron, a single measurement would include a large degree of measurement error and, as a consequence, observed association such as relative risk would be increasingly attenuated.

We found that high serum levels of ferritin and nitrite may be associated with a decreased risk of RCC.

Future study:

BAI is the fraction of iron responsible for inducing oxidant formation, as well as enhancing cancer cell proliferation. Therefore, measurements of BAI are expected to be a better indicator of iron carcinogenicity than the measurements of ferritin and transferrin which levels do not reflect the true bioactive iron exposure. The recent development of a fluorescent chemosensor of BAI by our laboratory now permits us to measure the levels of BAI in biological fluids such as sera and test the hypothesis that high levels of iron and estrogen may be associated with increased risk of breast cancer, particularly in post-menopausal women.

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BioMetals **0**: 1–9, 2002.

Calcein as a fluorescent iron chemosensor for the determination of low molecular weight iron in biological fluids

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Abstract

The fluorescence quenching of calcein (CA) is not iron specific and results in a negative calibration curve. In the present study, deferoxamine (DFO), a strong iron chelator, was used to regenerate the fluorescence quenched by iron. Therefore, the differences in fluorescence reading of the same sample with or without addition of DFO are positively and specifically proportional to the amounts of iron. We found that the same iron species but different anions (e.g. ferric sulfate or ferric citrate) differed in CA fluorescence quenching, so did the same anions but different iron (e.g. ferrous or ferric sulfates). Excessive amounts of citrate competed with CA for iron and citrate could be removed by barium precipitation. After optimizing the experimental conditions, the sensitivity of the fluorescent CA assay is 0.02 μM of iron, at least 10 times more sensitive than the colorimetric assays. Sera from 6 healthy subjects were tested for low molecular weight (LMW) chelator bound iron in the filtrates of 10 kDa nominal molecular weight limit (NMWL). The LMW iron was marginally detectable in the normal sera. However, increased levels of LMW iron were obtained at higher transferrin (Tf) saturation (1.64–2.54 μM range at 80% Tf saturation, 2.77–3.15 μM range at 100% Tf saturation and 3.09–3.39 μM range at 120% Tf saturation). The application of the assay was further demonstrated in the filtrates of human liver HepG2 and human lung epithelial A549 cells treated with iron or iron-containing dusts.

Abbreviations: CA – calcein; DFO – deferoxamine; FBS – fetal bovine serum; LMW – low molecular weight; NMWL – nominal molecular weight limit; NTBI – non-transferrin bound iron; RFU – relative fluorescence units; SI – serum iron; Tf – transferrin; TIBC – total iron binding capacity; UIBC – unbound iron binding capacity.

Introduction

Iron is present in animal cells in various forms, all bound to either low molecular weight (LMW) species, such as citrate, or as an integral part of proteins, which are segregated in the various cell compartments (Jacobs 1977). Iron is long thought to be the active metal species responsible for generating reactive oxygen species through Fenton or Haber-Weiss or iron autoxidation reactions (Comporti *et al.* 2002; Toyokuni 1996; Welch *et al.* 2002). Iron stored in the proteins is tightly bound, and thus not readily bioavailable for adverse effects. The labile iron pool of cells constitutes a cytosolic fraction of LMW bound iron, which can be characterized as iron (a) exchangeable and chelatable; (b) easily bioavailable for uptake by ferritin, heme, transferrin, or chelators; (c) metabolically and catalytically reactive for oxidant formation and likely responsible for iron toxicity; (d) possibly having regulatory properties which may affect iron responsive element-binding protein activity per se (Breuer *et al.* 1996; Miller *et al.* 1991). LMW iron in human body is not well defined and levels of LMW iron in serum have never been reported or estimated. Although several methods have been introduced for the detection of non-transferrin bound iron (NTBI) (Breuer *et al.*

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2000; Gosriwatana *et al.* 1999), this fraction of iron includes not only LMW iron, but also iron bound to other proteins such as albumins. Several epidemiological studies have shown that high levels of iron storage in the body or high iron intake through diet may have increased risk of developing cancer (Selby & Friedman 1988; Stevens *et al.* 1988; Wurzelmann *et al.* 1996). Possible explanations for this observation include oxidative DNA damage induced by high levels of LMW iron in the body as well as LMW iron-enhanced cancer cell proliferation. Clearly, it is important to quantify levels of LMW iron accurately in biological fluids.

The recent use of calcein (CA) as a fluorescence probe for LMW iron provides a major methodological breakthrough. The principle of the test is based on the binding of CA to LMW iron, in a stoichiometric manner, which results in a fluorescence quenching. Therefore, a lower reading in fluorescence intensity indicates a higher level of LMW iron in biological fluids. Calcein acetoxymethyl ester and Fl-DFO were used to estimate labile iron pool in cells or DFO chelatable iron, a component of serum NTBI (Breuer *et al.* 2001; Breuer *et al.* 2000; Cabantchik *et al.* 1996; Epsztejn *et al.* 1997; Petrat *et al.* 1999). However, this fluorescent CA assay has some technical disadvantages when applied to biological fluids such as serum. First, fluorescence of CA can be quenched by compounds in the serum, which are not iron-related. Such interference can greatly affect the specificity of the assay and its determination of iron levels. Second, citrate present in cell lysate or serum, if excessive, may compete with CA for LMW iron, thus leading to a decreased fluorescence quenching, big variations among assays, and sometimes, irreproducible results. Third, negative calibration curve between fluorescence reading and iron concentration could be confusing in comparison with the conventional positive standard curve. In the present study, we have improved the specificity of the assay by adding iron chelator DFO into the samples and the sensitivity of the assay by removing citrate as well as using appropriate standards. By filtering the sample through an Ultra-free membrane with nominal molecular weight limit (NMWL) of 10 kDa, the iron present in the filtrates should be LMW-bound. After optimizing the experimental conditions, we have shown that CA can detect LMW iron in biological fluids at a detection limit of 0.02 μM.

Materials and methods

Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), ferric sulfate pentahydrate [$Fe_2(SO_4)_3 \cdot 5H_2O$),] ferric citrate ($FeC_6H_5O_7$), sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), calcein (CA), deferoxamine mesylate (DFO), N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), barium chloride ($BaCl_2$), and sodium phosphate were purchased from Sigma (St. Louis, MO) with the highest purity available. Kits for serum iron (SI) and total iron binding capacity (TIBC) determination were also obtained from Sigma.

Sample preparation and fluorescence reading

All solutions were prepared in HPLC grade water except CA was first dissolved in 1 N NaOH solution at 20 mM and further diluted in HPLC water. All experiments were carried out in triplicates or quadruplicates in a Costar 96-wells microplate (Fisher Scientific, Pittsburgh, PA). Each well contained 80 μl CA (2 μM), 20 μl HEPES (500 mM), pH 7.4, and 20 μl distilled H_2O. Samples or iron standards (80 μl) were then added to the well. The plate was covered and incubated in a water bath at 37 °C for 20 min. The fluorescence was then determined on a fluorescence-chemiluminescence microplate reader (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA) with an emission at 515 nm and an excitation at 485 nm. To reverse iron-induced CA fluorescence quenching, 5 μl DFO (1 mM) or 5 μl H_2O were added to two of the quadruplicated samples. The reaction mixtures were incubated in a water bath at 37 °C for 10 min. The net fluorescence quenched by iron was obtained from the difference in fluorescence readings between with or without DFO ($\pm$ DFO). A plot of the difference in relative fluorescence unit (FRU) versus [Fe] can be generated, and a positive linear standard curve can be obtained for calculating levels of the LMW iron in biological fluids.

Citrate, a biologically relevant component in sera, may compete with CA for iron binding. To check this, high molar ratios of citrate/Fe^{3+} solutions (up to 500:1) were prepared by mixing ferric citrate (constant at 20 μM) with sodium citrate dissolved in HPLC H_2O. Following 30 min incubation at room temperature, the fluorescence quenching of CA by the mixtures were measured as described above. To remove the excessive citrate anions in the mixtures, $BaCl_2$ (final concentration 50 mM) was used to precipitate cit-

rate out at 4 °C. After 1 h to overnight incubation, the mixtures were centrifuged at 12,000 rpm for 10 min (Marathon MicroA Centrifuge), and the supernatants were asses. ed for CA fluorescence quenching.

Measurements of serum iron and total iron-binding capacity

Serum samples from healthy subjects were aliquoted in 1 ml in Ependorff tubes. Appropriate IBRA approval was obtained from New York University School of Medicine. Fat was removed by centrifugation. Serum samples were characterized before experiments using kits from Sigma Diagnostics. Techniques for determining both serum iron (SI) and serum unsaturated iron binding capacity (UIBC) and deriving the total iron binding capacity (TIBC) and transferrin saturation rate (%) are outlined as follows: At acidic pH (acetate buffer, pH 4.5) and in the presence of hydroxylamine (a reducing agent), transferrin-bound iron and LMW iron, if present in serum, dissociate to release ferrous ions. These react with ferrozine to form a stable magenta-colored complex (Fe^{2+}-ferrozine) with a maximum absorption at 560 nm. The difference in absorbance at 560 nm before and after ferrozine addition in serum sample is proportional to SI concentration. In contrast to SI, serum UIBC was measured at alkaline pH (TRIZMA®, pH 8.1). Ferrous ions added to the serum bind specifically with transferrin at unsaturated iron-binding sites and then remaining unbound ferrous ions are measured with the ferrozine reaction. The difference between the amount of unbound iron and the total amount added to serum is equivalent to the quantity bound to transferrin, which is the UIBC. The serum TIBC equals the SI plus the UIBC. Serum transferrin saturation rate (%) was calculated using SI divided by TIBC X 100.

Determination of LMW iron at different transferrin saturation rates

80, 100, and 120% transferrin saturation rate in serum samples were made by mixing 220 μl of serum with 220 μl of a given concentration of freshly prepared $FeSO_4$ solution. The amount of iron given was based on the data of the SI and TIBC of each individual sample. The mixtures were then incubated for overnight at 4 °C. The samples (0.4 ml) were filtered through ultra-free membrane with 10 kDa NMWL (Millipore, Bedford, MA) at 12,000 rpm for 2 h. Serum filtrates were added in quadruplicates (80 μl each) to CA (80 μl 2 μM) in Costar 96-wells plate containing 20 μl HEPES buffer (500 mM) and 20 μl distilled H_2O as previously described. After incubation at 37 °C for 20 min, the fluorescence was determined. Five μl DFO (1 mM) or H_2O were added to two of the quadruplicates and incubated at 37 °C for additional 15 min. The fluorescence was determined again and the net fluorescence quenched by iron was obtained from the data subtraction between with or without DFO addition.

Measurements of LMW iron in cells treated with $FeSO_4$ or coal dusts

Human liver HepG2 cells and human lung epithelial A549 cells were cultured in a 75-cm^2 flask (Fisher Scientific) in 10 ml of alpha Minimum Essential Medium (α-MEM) containing 10% fetal bovine serum (FBS), 1% antibiotics, 1% L-glutamine and grown in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. At approximately 70% confluence, HepG2 cells were treated with different concentrations of ferrous sulfates for 24 h. A549 cells were treated with two coal dusts without serum for 24 h. After treatment with iron or coal dusts, cells were washed with ice-cold phosphate-buffered saline and trypsinized. Cells were then counted and cell diameter measured by Coulter Particle Size and Analyzer (Beckman). Cells were finally suspended in 0.5 ml cold distilled water, and lysed by 6 cycles freeze-thaw in liquid nitrogen and in water bath at 37 °C. After centrifugation through a 10 kDa NMWL membrane at 4 °C, the filtrates were used for LMW iron determination. Levels of LMW iron were expressed as μM of iron in the filtrates or nanomoles iron per 10^6 cells or μM of iron within cells [estimated using mean cell volume (MCV)].

Results

CA fluorescence quenched by $FeSO_4$ and recovered by DFO

The quenching effect of Fe^{2+} ions ($FeSO_4$) on CA fluorescence was examined by measuring the changes in fluorescence intensity of CA after mixing various iron concentrations (0–1.6 μM range) with CA (0.8 μM final concentration) in 50 mM HEPES buffer, pH 7.4. Figure 1A shows that the CA fluorescence intensity was linearly decreased as increasing Fe^{2+} concentrations up to 0.4 μM $FeSO_4$, where the CA/Fe^{2+} molar ratio was 2:1. The fluorescence was regenerated when DFO was added. DFO alone can neither quench nor enhance the CA fluorescence intensity. Because

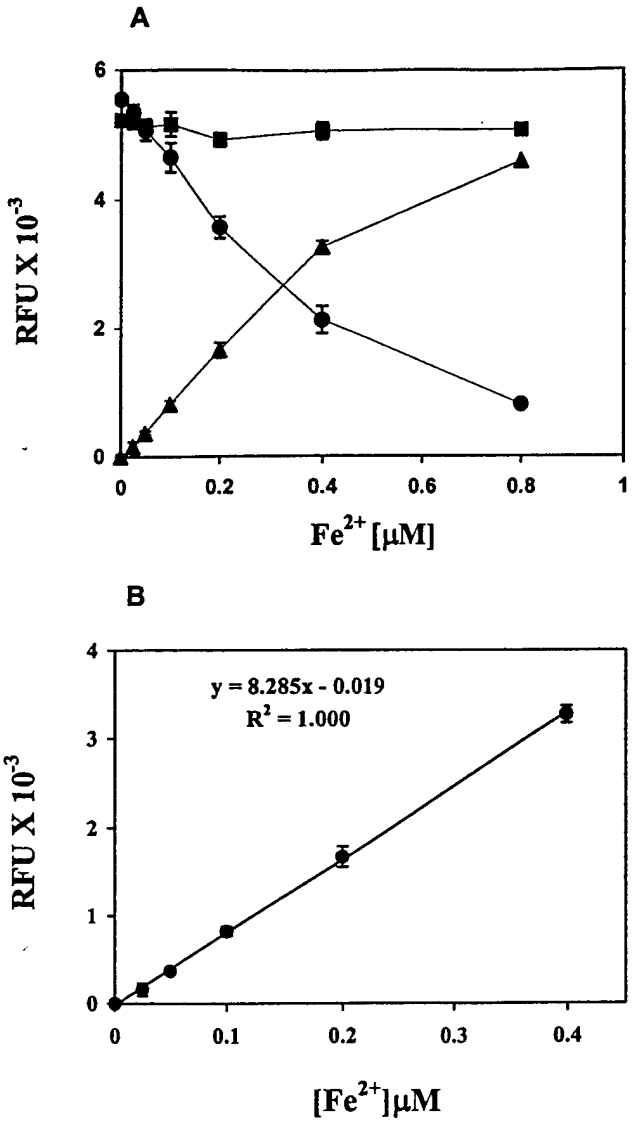

Fig. 1. CA fluorescence quenching by iron and its regeneration by DFO (A) and the differences in fluorescence reading between ± DFO as a function of iron concentrations (B). 80 μl of freshly prepared $FeSO_4$ solution were added to Costar 96-wells plate containing 80 μl CA (2 μM), 20 μl HEPES buffer (500 mM, pH 7.4), and 20 μl H_2O. The plate was incubated in a water bath at 37 °C for 20 min. The fluorescence was read on a microplate reader. 5 μl DFO (1mM) was added to each well and the fluorescence was read 10 min later. (A): •, before DFO addition; ■, after DFO addition; ▲, differences between ± DFO addition. (B) A positive and linear correlation between iron concentrations (0–0.4 μM iron) and differences in fluorescence reading of CA (0.8 μM final) between ± DFO. Data were means ± standard deviation (SD) of 12 determinations from three independent experiments.

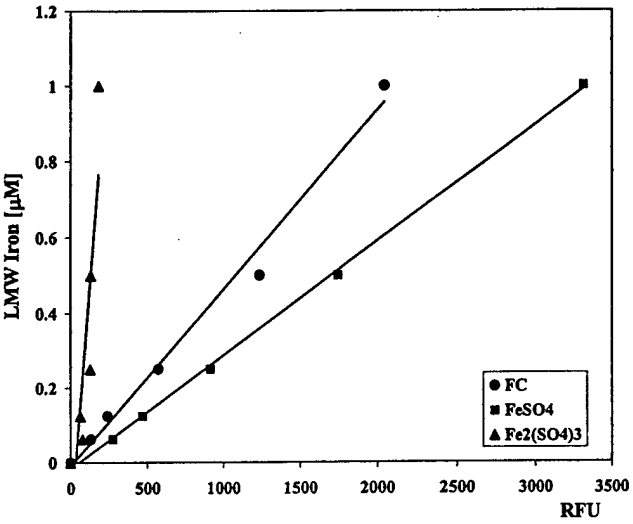

Fig. 2. Quenching effects of different iron compounds on the CA fluorescence. Data were means ± SD of three independent experiments. FC: ferric citrate. X-axis was the net fluorescence quenched by iron.

of the relative specificity of DFO for iron chelation, the differences in fluorescence reading between with and without DFO is related to the amounts of iron. Figure 1B shows a positive and linear correlation between the fluorescence differences (± DFO) and iron concentrations.

Various quenching abilities of different iron compounds

To compare the effects of Fe^{2+} and Fe^{3+} ions on CA fluorescence quenching, three iron compounds freshly prepared were used for the present study. Figure 2 shows that different iron compounds have different capacities to quench CA fluorescence. Among the three iron compounds tested, ferrous sulfate ($FeSO_4$) was the most active compound in diminishing CA fluorescence, followed by ferric citrate. Ferric sulfate had the weakest quenching effect.

Competition of citrate with CA for iron

Citrate anions are thought to be abundant in biological fluids, especially in serum (Grootveld *et al.* 1989; Jacobs 1977). Although citrate has a weaker binding affinity to iron than calcein does (Thomas *et al.* 1999), we tested a hypothesis that excessive amounts of citrate may compete with CA and thus, interfere the iron measurements. Figure 3 shows that CA fluorescence (2 μM) was quenched 80% by 2 μM Fe^{3+}

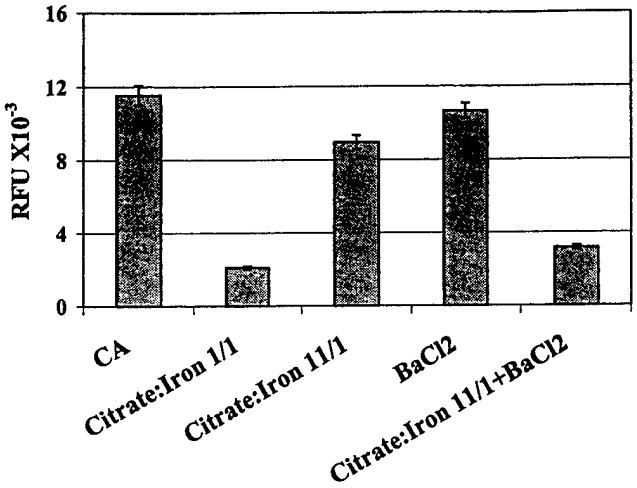

Fig. 3. Decreased CA fluorescence quenching by iron in the presence of excessive amounts of citrate and effects $BaCl_2$ on the removal of citrate. Citrate/Fe^{3+} mixtures with molar ratios ranging from 1:1 to 501:1 were prepared for CA fluorescence quenching. The mixture (citrate: iron 11:1) was incubated with $BaCl_2$ (50 mM) and then re-assessed for CA fluorescence quenching after centrifugation. Data points are means ± SD of 6 determinations from two independent experiments.

when the molar ratio of citrate/ Fe^{3+} was 1:1. Increasing the concentrations of citrate while keeping Fe^{3+} constant at 2 μM, we found that a molar ratio of citrate/ Fe^{3+} of 11:1 or higher significantly diminished the CA fluorescence quenching by the same concentration of Fe^{3+} ions. This was reflected as a higher reading of fluorescence in Figure 3. These results indicate that removal of citrate is necessary to improve the sensitivity of the assay. $BaCl_2$ was used to precipitate citrate (Ba citrate: water insoluble). We found that $BaCl_2$ itself had no quenching on CA fluorescence. At a final concentration of 50 mM, $BaCl_2$ significantly removed citrate from the mixture when the citrate/Fe^{3+} ratio was less than 80:1. This was shown in Figure 3 that lower reading of CA fluorescence by the mixture treated with $BaCl_2$ as compared to the same mixture without $BaCl_2$ treatment.

Interference of fluorescence quenching by non-iron related compounds in serum

Incubation of CA (2 μM) with sera in equal volume resulted in 60–70% of CA fluorescence quenching. DFO, even at higher concentrations and longer incubation periods than previously described in the *Methods*, did not restore the fluorescence. These results indicate that large parts of the CA fluorescence quenched by sera are caused by some components,

Table I. Levels of serum iron (SI), total iron binding capacity (TIBC), and Tf saturation (TS) in 6 healthy subjects.

Serum[a]	SI (μM)	TIBC (μM)	TS (%)
1	19.64 ± 0.38	52.70 ± 1.17	37.28 ± 1.19
2	19.01 ± 0.77	44.06 ± 0.98	43.17 ± 1.56
3	14.83 ± 1.34	48.13 ± 2.71	30.87 ± 2.03
4	15.81 ± 0.40	56.04 ± 1.69	28.20 ± 0.64
5	12.73 ± 1.31	65.29 ± 3.40	19.48 ± 1.00
6	17.57 ± 1.18	51.61 ± 4.69	34.16 ± 1.43

[a]Serum samples from 6 healthy individuals were collected on a monthly basis for three months and were combined for each individual. They were stored at −80 °C until analysis. Data presented were means ± SD of 9 determinations from three independent experiments.

which are not iron-related. After filtering sera through the 10 kDa ultra-free membrane, the fluorescence quenching component(s) in the sera were absent in the filtrates. We found that over 99% iron (Fe^{2+} or Fe^{3+} dissolved in water or buffer) can pass through the membrane. Because of this NMWL of 10 kDa, the filtrate is termed as LMW fraction, and iron, if present in the filtrate, is termed as LMW iron.

Characteristics of serum transferrin iron from six healthy subjects

In normal healthy individuals, essentially all circulating serum iron is bound to transferrin (Tf). The data shown in Table 1 indicated that both of SI and TIBC were in normal range but were varied between 12–19 μM for SI and 44–65 μM for TIBC. Regarding Tf saturation rate (%), great individual differences were observed among the 6 individuals, ranging from 19–43% saturated. LMW iron was marginally detectable in the ultra-free filtrates of the serum samples. After subtracting the blank, levels of LMW iron varied from 0.08±0.05 to 0.19±0.07 (SD) μM. These amounts of LMW iron may be truly from the serum samples, because the differences in fluorescence reading between ± DFO in the blank (CA in Hepes buffer) was about 100, representing about 0.016 μM iron.

Determination of LMW iron in sera of healthy individuals at high Tf-saturation

According to the Table 1 of the levels of SI and TIBC in serum of each individual, a given amount of $FeSO_4$ was added to each serum in order to obtain 80, 100, or 120% Tf saturation, respectively. After filtration, LMW iron was determined using CA assay and was

Table 2. Levels of LMW iron in the sera of 6 healthy subjects with different transferrin saturation (TS).

Serum[a]	LMW iron (μM)		
	80% TS	100% TS	120% TS
1	1.64 ± 0.04	2.78 ± 0.02	3.18 ± 0.11
2	1.66 ± 0.10	2.78 ± 0.11	3.18 ± 0.16
3	1.82 ± 0.39	2.77 ± 0.32	3.22 ± 0.15
4	2.26 ± 0.58	3.04 ± 0.52	3.09 ± 0.63
5	2.54 ± 0.13	3.15 ± 0.25	3.39 ± 0.13
6	1.92 ± 0.49	2.85 ± 0.05	3.26 ± 0.05

[a]The serum samples were the same as in the Table 1. According to the TIBC of each individual, transferrin were artificially saturated to the expected rate by adding $FeSO_4$ into the serum (details in the Methods). Data presented were means $\pm$ SD of 12 determinations from three independent experiments.

shown in Table 2. The LMW iron was consistently detected in all of 6 sera at 80% Tf-saturation, with concentrations ranging from 1.6 to 2.5 μM. Levels of LMW iron were increased as increasing Tf saturation. The average levels of LMW iron among the 6 individuals were 2.02 μM for 80%, 2.92 μM for 100%, and 3.25 μM for 120% Tf saturation rate, respectively.

Addition of $BaCl_2$ to the LMW fraction of serum filtrate did not result in neither precipitation nor higher fluorescence quenching, suggesting that citrate anions may not be excessive to the extent for competing with CA. To be prudent, the calibration curve using $FeSO_4$ was constructed in the control serum filtrates where an equation of [LMW iron] $=0.0005 \times$ [RFU] + B, $r = 0.99$, was obtained. RFU is the difference in relative fluorescence units (RFU) between $\pm$ DFO of the same sample. When the same iron standard tested in HPLC water, a linear calibration with equation of [LMW iron] $= 0.0003 \times$ [RFU] + B, $r = 0.99$ was obtained. These results indicate that the same amounts of iron in control serum filtrates have less quenching ability than in water, probably due to some anions present in serum, which competes with CA for iron binding. The calibration curve constructed in the control serum filtrates was used to quantify the levels of LMW iron in the sera of different Tf saturations described above.

Determination of LMW iron in cell culture

Table 3 shows that LMW iron in control HepG2 cells (grown in 10% FBS) was 0.074 ± 0.032 μM (mean $\pm$ SD, $n = 3$) within cells or 2.06 ± 0.50 nmoles/10^6 cells, levels of which cannot be measured by colorimetric methods. Levels of LMW iron were increased as increasing iron concentrations (Table 3). Higher level of LMW iron was obtained in control A549 cells grown in media without FBS during coal treatment (0.35 ± 0.02 μM within cells or 11.28 ± 0.56 nmoles/10^6 cells) (Table 4). The highest level of LMW iron was detected in the sample pre-treated with the coal dust from Pennsylvania (PA) coalmine region (2.41 ± 0.13 μM within cells or 78.59 ± 4.17 nmoles/10^6 cells at 20 μg/cm^2 treatment). Levels of LMW iron were dose-dependent in samples treated with the PA coal but not with the Utah (UT) coal (Table 4). At higher doses, the coal from UT also significantly increased levels of LWM iron. Prior to cell treatment, we have measured that levels of bioavailable Fe^{2+} and Fe^{3+} in the coals were 2786.4 parts per million (ppm) and 7244.1 ppm for the PA coal, and 19.3 ppm and 25.8 ppm for the UT coal, respectively (Huang *et al.* 1998; Zhang *et al.* 2002). Levels of LMW iron in A549 cells correlated with the levels of bioavailable iron in the coals. Interestingly, prevalence of pneumoconiosis also paralleled with the levels of LMW iron in the A549 cells (Morgan *et al.* 1973).

Discussion

In comparison with our detailed knowledge of iron proteins and their regulations (Addess *et al.* 1997; Aisen *et al.* 1999; Theil 1990, 1998), the knowledge on the toxicological aspect of LMW iron is very limited. For example, it has been thought for a long time that tissue damage in iron overload is through LMW iron-mediated oxidative stress mechanism and iron trapped in iron proteins is not readily available for adverse effects (Huang *et al.* 2002; Kang *et al.* 1998). However, the amounts of LMW iron in healthy subjects have never been estimated, possibly due to the detection limits of the assays available. Quantification as well as clarification of the chemistry of the LMW iron will give a better insight into intracellular iron metabolism, and thereby, into a number of pathologic mechanisms induced by iron-related diseases.

The assay in the present study is based on the iron sensitive fluorescence probe CA, which fluorescence can be quenched upon binding to iron. The nature of the iron species, whether Fe^{2+} or Fe^{3+} can quench CA fluorescence, were debated and carefully studied (Thomas *et al.* 1999). Under our experimental conditions, we found that both Fe^{2+} and Fe^{3+} can quench CA fluorescence, but with different quenching ability depending upon the ligands as shown in Figure 2.

Table 3. Levels of LMW iron in human liver HepG2 cells treated with iron compound[a].

Samples	Treatments	LMW iron (μM in the filtrate)	LMW iron (nmol/10^6 cells)	LMW iron (μM within cells)[b]
1	Control[c]	0.075 ± 0.012	2.06 ± 0.50	0.074 ± 0.032
2	50 μM Fe^{2+}	0.097 ± 0.024	4.47 ± 0.60*	0.112 ± 0.044
3	100 μM Fe^{2+}	0.117 ± 0.038	5.23 ± 0.62**	0.155 ± 0.026*
4	200 μM Fe^{2+}	0.166 ± 0.056	7.49 ± 0.55**	0.215 ± 0.036**

[a]HepG2 cells were treated with $FeSO_4$ for 24 h in the presence of 10% FBS and then lysed by freeze-thaw. After ultra-free centrifugation (10 kDa), LMW iron in the filtrates was determined using differences in fluorescence reading between ± DFO of the same sample. Data presented were means ± SD of three independent experiments.
[b]Estimated using mean cell volume.
[c]Control samples were cells without any treatments.
*Significantly different from control by Student's t-test ($P < 0.05$).
**Significantly different from control by Student's t-test ($P < 0.01$).

Table 4. Levels of LMW iron in A549 cells treated with coal dusts from Pennsylvania (PA) and Utah (UT)[a].

Treatments	LMW iron (μM in the filtrate)		LMW iron (nmol/10^6 cells)		LMW iron (μM within cells)[b]	
	UT	PA	UT	PA	UT	PA
Control[c]	0.118 ± 0.005	0.118 ± 0.005	11.28 ± 0.56	11.28 ± 0.56	0.35 ± 0.02	0.35 ± 0.02
2 μg/cm^2	0.121 ± 0.010	0.184 ± 0.014	11.28 ± 0.93	26.76 ± 2.07*$^{\oslash}$	0.35 ± 0.03	0.82 ± 0.06*$^{\oslash}$
5 μg/cm^2	0.120 ± 0.012	0.235 ± 0.012	10.28 ± 1.08	27.95 ± 1.44*$^{\oslash}$	0.32 ± 0.03	0.86 ± 0.04*$^{\oslash}$
10 μg/cm^2	0.122 ± 0.009	0.270 ± 0.013	13.36 ± 0.99*	39.56 ± 1.65*$^{\oslash}$	0.41 ± 0.03*	1.21 ± 0.05*$^{\oslash}$
20 μg/cm^2	0.130 ± 0.012	0.635 ± 0.032	13.40 ± 1.23*	78.59 ± 4.17*$^{\oslash}$	0.41 ± 0.04*	2.41 ± 0.13*$^{\oslash}$

[a]A549 cells were treated with two coal dusts for 24 h, in the absence of FBS, one from PA coalmine region, and the other from UT. LMW iron was determined as described in the legend of Table 3.
[b]Estimated using mean cell volume.
[c]Control samples were cells without any treatments. Data on LMW iron were average of three independent experiments ± SD.
*:Significantly different from control by Student's t-test ($P < 0.05$)
$^{\oslash}$:Significantly different from UT by Student's t-test ($P < 0.05$)

Therefore, appropriate iron compound should be used for the calibration curve. Ferric citrate is likely the most appropriate iron standard for the determination of LMW iron because of its dominance in biological samples.

DFO was added to the filtrates, which regenerated the CA fluorescence. DFO can bind to Fe^{3+}, as well as Al^{3+} and Ga^{3+}. However, Al^{3+} and Ga^{3+} cannot quench CA fluorescence (data not shown). Moreover, neither Al^{3+} nor Ga^{3+} ions are likely to be found in serum at nanomolar or micromolar concentrations. Cu^{2+} can also efficiently quench CA fluorescence, but the quenching part cannot be regenerated by DFO. Thus, the fluorescence regenerated by DFO in the assay is solely due to iron. The differences in fluorescence reading of the same sample between ± DFO specifically quantify the LMW iron, as shown by a linear correlation in Figure 1B. Adding DFO to the filtrates showed a much faster kinetics in regenerating CA fluorescence (5–10 min incubation) than when serum was present (2 h or longer) (Grootveld *et al.* 1989).

By filtering the samples through a 10-kDa membrane, the iron present in the filtrate should be LMW bound. According to the manufacturer (Millipore), this 10-kD membrane can eliminate 95–100% of bovine serum albumin (molecular weight limit 67 kD) and 80–90% lysozyme (14 kD) at 25 °C. In contrast, 90–95% of vitamin B-12 (1.3 kD) can be recovered in the filtrate. The binding affinity of CA to iron is between those of citrate and EDTA (Thomas *et al.* 1999). Therefore, the LMW iron measured by CA fluorescence should be redox active, and thus biological relevant, particularly in the context of iron-mediated oxidative stress.

Since citrate can compete with CA, removal of citrate might be necessary to have reliable measurements of LMW iron with greater sensitivity. However, when adding $BaCl_2$ to the serum filtrates, no precipitation was seen, suggesting that levels of citrate in the serum samples were not sufficient enough for a precipitation by 50 mM $BaCl_2$. Therefore, we would not recommend adding $BaCl_2$ into the serum filtrates, though the calibration curve in the control serum filtrates should be used to quantify the levels of LMW iron in serum.

Levels of LMW iron in the filtrates of the 6 healthy subjects ranged from 0.09 to 0.19 μM, after subtracting the blank. Differences in fluorescence reading between the blank sample (CA in Hepes buffer only) $\pm$ DFO were approximately 100 RFU, representing 0.016 μM iron in the CA solution (according to Figure 1B). Therefore, the results in the filtrates of control sera may be meaningful, although no detectable levels of LMW iron were previously reported in serum samples of healthy controls (Breuer *et al.* 2000, 2001; Breuer, Ronson *et al.* 2000; Grootveld *et al.* 1989; Porter *et al.* 1996). Increased levels of LMW iron were observed in the filtrates of sera by artificially saturating transferrin at 80, 100, and 120% rate, respectively (Table 2). Since these serum samples were iron saturated under well-controlled conditions, much lower levels of LMW iron than expected were detected at 120% Tf saturation. These results indicate that LMW iron measured by CA after filtration represents only a fraction of NTBI, which is about 30% under our experimental conditions (calculated based on the TIBC and LMW iron at 120% saturation). NTBI is probably comprised of heterogeneous forms of iron complexes. For example, we have previously shown that iron, as well as nickel, can non-specifically bind to proteins isolated from heterochromatin (Huang *et al.* 1995).

To further validate our assay, levels of LMW iron in cells treated with iron or iron containing coal dusts were measured (Tables 3 and 4). Again, increased levels of LMW iron were observed in HepG2 cells treated with pure iron compounds. The presence of 10% FBS in HepG2 cell media may chelate iron and thus, result in lower background level of LMW iron in control HepG2 cells than that in control A549 cells grown without FBS during coal treatment (Tables 3 and 4). A statistically significant increase in LMW iron was also observed in A549 cells treated with the coal from PA coalmine region. The coal from PA with a high prevalence of coal workers' pneumoconiosis (26%) released high levels of LMW iron (Attfield & Morring 1992; Huang *et al.* 1998; Morgan *et al.* 1973). The coal from UT with a low prevalence of pneumoconiosis (4%) had low levels of LMW iron. These results suggest that LMW iron released by various coal samples may contribute to the development of pneumoconiosis.

In summary, CA is a sensitive fluorescent iron chemosensor, which can be applied for the measurements of LMW iron. The assay is simple and can be iron specific. Ferric citrate is probably the most appropriate iron standard for CA fluorescence quenching. The differences in fluorescence reading between the same sample $\pm$ DFO are recommended for iron quantification. After optimizing the experimental conditions, the sensitivity of the CA assay is 0.02 μM of iron, at least 10 times more sensitive than the colorimetric assays.

Acknowledgements

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Cancer Epidemiol. Biomark. Prev.

Reliability of serum iron, ferritin, nitrite, and association with risk of renal cancer in women

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Running Title: Renal cancer risk in women

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Abstract:
Reliability of serum levels of iron, ferritin and nitrite (NO_2^-) over a two-year period were evaluated in 40 healthy women (20 pre- and 20 post-menopausal), ages 39-65 years, from the New York University Women's Health Study (NYUWHS). Three blood samples per woman collected at yearly intervals were analyzed. Reliability coefficients of serum iron, ferritin, and nitrite were 0.03 [95% confidence interval (CI), 0-0.33], 0.90 (95% CI, 0.79-0.95), and 0.72 (95% CI, 0.50-0.86), respectively for pre-menopausal women, and 0.26 (95% CI, 0-0.56), 0.77 (95% CI, 0.59-0.89), and 0.55 (95% CI, 0.30-0.77), respectively for post-menopausal women. In a case-control study nested within NYUWHS cohort, serum levels of nitrite, ferritin, and iron were measured in women apparently healthy at the time of blood donation but diagnosed with renal cancer 1.8-12.2 years later (n = 24) and in individually matched controls (two per case). The results suggest that high serum levels of ferritin and nitrite may be associated with a decreased risk of renal cancer [odds ratio (OR), 0.55, 95% CI, 0.15-2.01 for ferritin, and OR, 0.52, 95% CI, 0.17-1.60 for nitrite in women with above median level as compared to women with below median level]. The possible role of ferritin and nitrite in renal cancer is discussed.

Key words: Renal cancer, ferritin, nitrite, serum iron, and reliability.
Abbreviations: NYUWHS: New York University Women's Health Study; RCC, renal cell carcinoma; TMB: Tetramethylbenzidine.

Introduction:

Kidney cancer accounts for 2.1 % of all cancer in men and 1.6 % in women. A steady increase in the number of patients with renal cancer has recently been noted, such that the rates for both men and women were 50% higher in the mid-1990's than the comparable rates in the early 1970's (1, 2). The American Cancer Society estimates that there should be about 30,800 new cases of renal cancer (18,700 in men and 12,100 in women) in the United States in the year 2001, and about 12,100 people (7,500 men and 4,600 women) will die from this disease (3). Renal cell carcinoma (RCC) accounts for 80-85% of all kidney cancers in the U.S. The remaining 15-20% of renal cancer are mostly cancers of the renal pelvis, which are anatomically and histologically distinct from RCC.

Cigarette smoking and obesity are established risk factors for RCC (4-6). Hypertension, medications such as thiazides, as well as occupational exposure to substances such as asbestos, cadmium, and solvents have been linked to an increased risk of RCC (7-9). Among dietary factors, an inverse relationship between risk and consumption of vegetables and fruits has been found (10). After accounting for the exposure to these risk factors, about half of RCC cases still remain unexplained (4).

The goal of the present study was to identify endogenous risk factors that may be involved in RCC development. Since kidney is one of the main organs for iron metabolism, we explored the hypothesis that high serum levels of iron, ferritin, and nitrite in the body may contribute to the etiology of RCC. It is well accepted that iron serves as a nutrient for cancer cell proliferation and that it causes oxidative DNA damage through its interaction with oxygen and hydrogen peroxide (11, 12). Several prospective studies have shown a positive association between high body iron stores and increased risk of cancer in general (13, 14). Although other studies reported either no association or an inverse association (15, 16), this discrepancy may be due to the fact that the iron measured in these studies represents only a small faction (<1%) of the total iron in the body (17). Serum iron, which is the iron stored in transferrin, total iron-binding capacity, which is the total level of transferrin when 100% saturated with iron, and percentage saturation of transferrin were used as biological indices of body iron storage in these studies (13-16, 18, 19). In contrast, ferritin, an iron storage protein, has rarely been measured. Because of its ability to sequester up to 4,500 molecules of iron per molecule ferritin, ferritin was postulated to have antioxidant properties, which may play a protective role in cancer development (20, 21). Nitrite is one of the end-products of nitric oxide, a messenger molecule playing a pivotal role in the physiological and patho-physiological regulation of many genes in various organs. It was found that the higher the RCC tumor grade, the lower the nitric oxide synthase activity, suggesting that nitric oxide may also be involved in RCC development (22).

Using serum samples collected by the New York University Women's Health Study (NYUWHS), a prospective cohort study of hormonal and environmental factors and cancer in women (23, 24), we have examined 1) the reliability of serum iron, ferritin, and nitrite measurements over a 2-year interval in 40 healthy women; 2) the association of serum levels of iron, ferritin, and nitrite with renal cancer risk in the 24 incident RCC cases diagnosed among all cohort participants after enrollment and 48 individually matched control subjects. Because the blood samples were drawn before clinical diagnosis of the RCC, the parameters that we measured were less likely to be influenced by the presence of the tumor.

Materials and Methods:

Subjects:

The NYUWHS is a prospective study of 14,275 women 34-65 years of age who attended the Guttman Breast Diagnostic Institute, a breast cancer-screening center in New York City, between March 1985 and June 1991 (23-25). Women were classified as post-menopausal at entry if they reported any one of the followings: 1) no menstrual cycles during the 6 months preceding enrollment; 2) total bilateral oophorectomy; 3) age of 52 or above and history of hysterectomy without total bilateral prior to natural menopause. Women who had taken hormonal medications in the 6 months preceding their visit were also not eligible. After written informed consent was obtained, demographic, medical, anthropometric, reproductive, and dietary data were collected using self-administered questionnaires. Thirty milliliters of non-fasting peripheral venous blood was drawn at enrollment and follow-up visits. After centrifugation, serum samples were divided into 1-ml aliquots and immediately stored at –80°C for subsequent biochemical analyses. About 50% of the subjects have had at least one follow-up visit.

Reliability study:

NYU Women's Health Study participants who had given blood on three or more occasions with a yield of 11 or more aliquots per visit, who had not been diagnosed with cancer or cardiovascular disease, and who had not been selected as a control in any case-control study nested within the cohort were eligible for the reliability study. One hundred-twenty serum samples collected at yearly intervals from 40 healthy women (20 pre-menopausal and 20 post-menopausal) were used for the reliability analysis. Levels of serum iron, ferritin, calcium (Ca), nitrite (NO_2^-), and total nitrite ($NO_2^- + NO_3^-$) were initially measured in 45 serum samples from 15 subjects (8 pre-menopausal women and 7 post-menopausal). The reliability coefficients (RCs) for serum iron, ferritin, Ca, nitrite, and total nitrite were 0.47, 0.77, 0.33, 0.51, and 0.06, respectively. For parameters with RCs>0.40, the study was extended to a total of 40 subjects in order to increase the precision of the reliability estimates. Ca and total nitrite were not studied any further.

Nested case-control study:

Cases of RCC were identified through the active follow-up of the cohort by questionnaires mailed approximately every 2 years and telephone interviews for non-respondents, as well as by record linkages with the state-wide cancer registries in New York, New Jersey, Connecticut, and Florida and with the U.S. National Death Index. Medical and pathology reports were requested to confirm the diagnosis. For each case subject, 2 controls were selected at random from the risk set of women who were alive and free of disease at the time of diagnosis of the case (index date), and who matched the case on menopausal status at the enrollment, date of birth (± 6 months), and number (1, > 1) of blood donations.

Laboratory analyses:

Fat was separated from the sera by centrifugation at 12,000 rpm for 15 min (Marathon MicroA Centrifuge, Fisher Scientific, Pittsburgh, PA). The clear serum from the lower part of the tube was carefully transferred to an Eppendorf tube with a 1-ml tuberculin syringe (Beckton Dickinson and Company, Franklin Lakes, NJ) and stored frozen at –80°C until analyses. The serum was thawed just before use by incubation on ice for 30 min and all assays were performed in 96-well microplates.

Serum iron was determined by Fe^{2+}-ferrozine complex formation using a diagnostic kit from Sigma Chemical Co. (St. Louis, MO). In brief, 30 µl serum samples were added in duplicates to wells containing 135 µl of serum iron buffer. After incubation at 37°C for 10 min, the

absorbance was determined at 560 nm using a UV-visible microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA). Five μl of iron color reagent (ferrozine) were then added, the plate incubated (37°C for 15 min), and absorbance measured at 560 nm. Iron concentration was determined from the iron standard curve constructed within the same microplate.

Serum Ca was measured at 600 nm using Arsenazo III (a diagnostic kit from Sigma). Following Manufacturer's recommendation, 10 μl serum samples were used for Ca determination.

Ferritin in sera was determined according to a previously published protocol (26). In brief, an antibody to a mixture of human spleen and liver ferritin was used as the capture antibody to coat an ELISA plate. Human liver ferritin was used as the standard (Roche Molecular Bio-Chemicals (Indianapolis, IN). The conjugate of peroxidase and antibody to human spleen and liver ferritin was then added to serve as the detector to determine the amount of ferritin bound to the capture antibody. Tetramethylbenzidine (TMB) was then added as the peroxidase substrate, and the absorbance of the peroxidase-mediated TMB oxidation product was determined at 450 nm using the UV-visible microplate reader.

Nitrite (NO_2^-) was measured using Griess reagents as previously described (27). Sixty μl of serum samples were used. After adding 50 μl 1% sulfanilamide (Griess reagent 1), followed by the addition of 50 μl 0.1% *N*-(1-napthyl)ethylenediamine (Griess reagent 2), the absorbance was determined at 540 nm after 10 min incubation. To determine levels of nitrate (NO_3^-) in serum, nitrate reductase (Roche Molecular Bio-Chemicals) was used to reduce nitrate to nitrite, and levels of total nitrite were then determined by Griess reagents as described above.

As a quality control measure of the assays, quadruplicates of one laboratory control serum sample were tested within the same microplate. All inter- and intra-assay laboratory coefficient of variations (CVs) for nitrite, ferritin, serum iron, and Ca measurements were $\leq$ 12%.

Statistical methods:

The reliability was estimated by the intraclass correlation coefficient. Variance components were obtained in an ANOVA analysis assuming a one-way random effects model. Reliability coefficients (RCs) were computed using the log-transformed data. Exact 95% confidence intervals (CI) were calculated (28). In the nested case-control study, the non-parametric Wilcoxon rank-sum test was used to test for differences in continuous variables between cases and controls. To compute odds ratios (OR), serum measurements were categorized into above or below median level using the distribution of the cases and controls combined. The data were analyzed using the conditional logistic regression model, which is appropriate for a matched case-control study design. The group with below median level was used as the reference group. Analyses were also performed on the continuous variables. Likelihood ratio tests were used to assess statistical significance. All *P* values are two-sided and *P* values <0.05 were considered statistically significant. Because of the known association between obesity and iron levels, we conducted analyses adjusting for body mass index reported at time of blood donation. The effect of other potential confounders was explored by addition of the potential confounders in the logistic regression models. Besides body mass index, the final adjusted models included smoking history.

Results:

Pre-menopausal women had a mean age (±SD) at first blood donation of 45.6 years (±4.4 years) and a mean body mass index of 25.6 kg/m^2 (±4.2 kg/m^2). For post-menopausal women, the mean age was 59.9 years (±2.3 years) and the means body mass index was 24.9 kg/m^2 (±5.0 kg/m^2). Mean times in storage of the serum samples were 15.1 years (±0.5 years), 14.1 years (±0.5 years), and 13.1 years (±0.6 years) for visits one, two, and three, respectively.

Table 1 shows that average levels at first visit of serum iron, ferritin, and nitrite were higher in post-menopausal women than in pre-menopausal women, and the difference was statistically significant for serum iron ($p<0.05$) and approaching significance for ferritin ($p<0.08$). At visits two and three, the same trends were observed (data not shown). Table 2 shows the RCs for the three parameters measured in the sera of all 40 subjects, confirming relatively high RCs for ferritin and nitrite as previously obtained using sera from 15 of the 40 subjects. Ferritin has the highest RCs, followed by nitrite, with higher RCs in pre-menopausal than in post-menopausal women. In contrast, serum iron has a low RCs, particularly in pre-menopausal women. This may be due to the variation in blood loss by menstrual cycling in pre-menopausal women.

Serum levels of iron, ferritin, and nitrite were then measured in 24 women who developed renal cell carcinoma (RCC) after enrollment in the cohort and 48 controls matching the cases on the age, menopausal status, and time of blood donation. The mean age ($\pm$SD) of the study population at blood donation was 56.9 years ($\pm$6.3 years). The ethnic distribution was as follows: 67% Caucasians, 8% African-Americans, and 25% others. The mean body mass index was 25.4 kg/m^2 ($\pm$3.6 kg/m^2) for case participants and 25.2 kg/m^2 ($\pm$4.0 kg/m^2) for control participants. Among the cases, the median lag time between blood donation and diagnosis of RCC was 6.6 years (range, 1.8-12.2 years). There was a higher proportion of ever smokers in cases (53%) than in controls (37%) with smoking status missing for 5 cases and 7 controls. As shown in Table 3, levels of serum iron, ferritin, and nitrite were slightly lower in the case than in the control subjects, but the differences were not statistically significant. The differences in levels of ferritin were somewhat larger between cases and controls in the analyses restricted to Caucasian women [average levels of ferritin in cases: 5.0 ng/ml (range 0.1-21.9, n=20) *vs* control: 6.0 ng/ml (range 0.5-27.1, n=28), $p<0.35$]. No differences in serum iron or nitrite were observed in analysis limited to Caucasian women. In conditional logistic regression analyses, higher levels of ferritin appeared to be associated with a decrease in risk (adjusted OR = 0.55 in the above median *vs* the below median) and nitrite (OR = 0.52 in the above median *vs* the below median) after adjustment for smoking and body mass index (Table 4). ORs in the above median *vs* below median seemed further decreased to 0.28 (95% CI: 0.05-1.45) and 0.34 (95% CI: 0.07-1.58) for serum ferritin and nitrite in Caucasian women after adjusting for smoking. A slight increase in risk appeared for serum iron (OR = 1.12 in the above median *vs* the below median).

Discussion:

The data in the present study indicate that, for ferritin, a single measurement may be sufficient to estimate long-term average level in a woman for epidemiological studies. Results from our nested case-control study suggest that risk of RCC may be inversely associated with ferritin and nitric oxide production as measured by serum ferritin and nitrite concentrations, respectively.

To our knowledge this is the first study to investigate the relationships between pre-diagnostic serum levels of iron, ferritin, nitrite, and risk of RCC later in life. The advantage of the case-control study nested within a prospective cohort is that case and control subjects originate from the same, well-defined source population, thereby minimizing the risk of selection bias. Furthermore, prospective studies offer the advantage that serum samples are obtained before the clinical manifestation of disease, therefore the observed association are less likely to be due to the effect of disease. The limitations of our study included its small sample size, resulting in a limited statistical power of the study, as well as the fact that data were based on one measurement from a single serum sample per individual. Therefore, caution is required in the interpretation of the results.

Ferritin is an iron storage protein found in all living organisms including mammals, bacteria, and plants (29). Its ability to sequester iron gives ferritin the dual functions of iron detoxification with antioxidant properties and iron reserve with pro-oxidant properties. The role of ferritin in cancer is not fully understood. Our results suggest that low levels of serum ferritin may result in high levels of bioavailable iron, forming oxidants that lead to DNA damage, as well as serving as a nutrient for cancer cell proliferation, and thus contributing to RCC development. The lower levels of ferritin in RCC cases than in matched controls may be due to the degradation of ferritin by endogenous or exogenous substances, such as estrogen metabolites, potentially leading to an uncontrolled release of iron from ferritin (30, 31). It has been shown that an elevated stomach cancer risk is associated with low serum levels of ferritin, with more than a three-fold excess among those in the lowest compared with the highest quintile (32). Our findings of a possible protective role of ferritin in RCC are in an agreement with this observation.

It is noteworthy that ferritin has been proposed as a clinical marker for staging and predicting survival of RCC, particularly in the case of recurrence after surgical therapy (33, 34). It was shown that the mean serum ferritin level from the renal vein correlated with tumor stage and was significantly higher than that from the peripheral vein (34). The mean cytosolic ferritin level of cancer tissue was also much higher than that from normal parenchyma (35, 36). However, the actual reasons for the ferritin increases in the RCC tissue remain unclear (36). In our study, blood samples were collected on average 6.6 years before clinical diagnosis of RCC, suggesting that low serum ferritin in the RCC cases may be due to the translocation of ferritin from the circulatory system (blood) to the pre-cancerous tissue (kidney). This hypothesis is plausible because kidney is one of main organs for iron metabolism. If this is the case, it indicates that the translocation of ferritin from the blood to the renal tissue may have happened years before clinical diagnosis of RCC.

Nitric oxide synthase catalyzes the oxidative conversion of L-arginine to NO and L-citrulline through a NADPH-dependent reaction. In experimental studies, NO formation is estimated by determining the amount of its end-products nitrate (NO_3^-) and NO_2^-, because NO is short-lived (half life, 10-60 seconds). The lower levels of nitrite in sera of RCC cases than those of controls are consistent with the previous observation that NOS activity was lower in RCC tissue than in non-malignant kidney tissue, and the higher the tumor grade, the lower the NOS activity (22). Several studies suggest that NO may play a protective role in RCC, possibly contributing to interleukin-2-induced antitumor activity, an immunotherapeutic agent for RCC treatment (22, 37, 38).

Our studies indicate that serum iron has a low reliability coefficient, particularly in pre-menopausal women. With greater variability of serum iron, a single measurement would include a large degree of measurement error and, as a consequence, observed association such as relative risk would be increasingly attenuated. Therefore, the low reliability coefficient of serum iron, as shown here, may contribute to the apparent discrepancies of the results observed in previous studies (13-16, 18, 19).

In conclusion, we found that high serum levels of ferritin and nitrite may be associated with a decreased risk of RCC. Further investigation in larger epidemiological studies appears warranted.

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Table 1. Average levels of serum iron, ferritin, and nitrite from 20 pre- and 20 post-menopausal women at first visit

	Pre-menopausal (n=20)		Post-menopausal (n=20)		
	Mean	(SD)	Mean	(SD)	*p*
Serum iron (μM)	12.36	4.48	14.93	3.57	0.05
Ferritin (ng/ml)	8.55	7.72	13.37	9.34	0.08
Nitrite (μM)	8.36	5.30	8.48	8.78	0.96

Table 2. Reliability coefficients (RCs) and 95% CI for each serum based on three yearly blood samples from 20 pre- and 20 post-menopausal women

	Pre-menopausal (n=20)		Post-menopausal (n=20)	
	RCs	95% CI	RCs	95% CI
Serum iron (μM)	0.03	0-0.33	0.26	0-0.56
Ferritin (ng/ml)	0.90	0.79-0.95	0.77	0.59-0.89
Nitrite (μM)	0.72	0.50-0.86	0.55	0.30-0.77

Table 3. Serum characteristics of RCC cases and controls, NYU Women's Health Study, 1985-2000

	Cases (n=24)		Controls (n=48)		
	Median	Range	Median	Range	P value*
Serum iron (μM)	12.0	5.5-24.9	12.6	3.3-25.3	NS**
Ferritin (ng/ml)	5.3	0.1-22.7	5.8	0-31.0	NS**
Nitrite (μM)	40.4	9.2-93.2	42.1	4.3-112.7	NS**

* Wilcoxon Rank Sum Test.
** Not significant (p-value > 0.05).

Table 4. OR and 95% CI for the association of above the median compared to below median levels of serum iron, ferritin and nitrite with risk of RCC, NYU Women's Health Study, 1985-2000

	Cases	Controls	Unadjusted*		Adjusted**	
	N	N	OR	95% CI	OR	95% CI
Serum iron (μM)						
Below Median (12.6 μM)	15	25	1.00		1.00	
Above Median	9	23	0.59	0.17-1.79	1.12	0.24-5.17
Ferritin (ng/ml)						
Below Median (5.8 ng/ml)	14	25	1.00		1.00	
Above Median	10	23	0.78	0.29-2.08	0.55	0.15-2.01
Nitrite (μM)						
Below Median (42.1 μM)	13	24	1.00		1.00	
Above Median	11	24	0.87	0.35-2.15	0.52	0.17-1.60

* Except for matching factors (age, menopausal status, and date of blood donation).
** Adjusted for smoking (never, ever) and body mass index (continuous variable).

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Serum ferritin, iron, and nitrite as risk factors of renal cancer in women

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Iron bound to low molecular weight (LMW) biomolecules is long thought to play an important role in human cancer. Carcinogenic effects of LMW iron can be due to 1) production of oxidants through interaction of iron with oxygen and/or hydrogen peroxide, which lead to DNA damage; 2) serving as a nutrient for cancer cell proliferation. Because there are no sensitive assays available for the detection of LMW iron *in vivo*, ferritin, an iron storage protein, can be used as an indicator of LMW iron. One molecule of ferritin has the capacity of binding up to 4,500 molecules of iron. Its ability to sequester iron gives ferritin the dual functions of iron detoxification with antioxidant properties and iron reserve. Although several epidemiological studies showed a positive association between high body iron stores and the risk of cancer, the role of ferritin in cancer is still unclear. In the present study, we have measured ferritin protein, serum iron, which is transported by transferrin, and nitrite (NO_2^-), a relatively stable end-product of nitric oxide synthase, in 120 sera samples collected at three different yearly intervals from 40 healthy women (20 pre-menopausal and 20 post-menopausal). Reliability coefficients (RC) of these parameters were then calculated. RC is the proportion of the variance due to between-subject variability and indicates how well a single measurement represents the long-term average level within an individual. We have found that ferritin and nitrite are more reliable (RC= 0.87 and 0.61, respectively) than SI (RC= 0.13) for epidemiological studies utilizing serum samples. Since the kidney is one of the main organs for iron metabolism, the roles of ferritin, SI, as well as nitrite, in renal cancer were investigated in a case-control study nested within the New York University Women's Health Study cohort. Serum levels of ferritin, nitrite, and iron were measured in women apparently healthy at the time of blood donation but who were diagnosed with renal cancer 6.6 years (range 1.8-12.2 years) later (n = 24) and their matched controls (n = 48). In the preliminary study, we have found that high prediagnostic serum levels of nitrite and ferritin may be associated with decreased risk of renal cancer. The role of potential confounders such as race, education, body mass index, smoking, regular alcohol use, and physical activity is being explored. Our findings suggest that ferritin and nitrite may play a protective role in renal cancer development. Supported by a grant from the Department of Defense DAMD17-01-10576